Does Integrin-Mediated Cell Death Confer Tissue Tropism in Metastasis?

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Abstract

To develop metastatic capability, tumor cells must evolve the capacity to survive in novel microenvironments. Recently, we showed that metastasis of neuroblastoma cells is enhanced by loss of caspase-8, an event that occurs frequently in this malignancy. In poorly metastatic cells, unligated integrins were found to trigger activation of caspase-8, providing a selective pressure to promote its attenuation and thereby increased survival in foreign adhesive environments. Our findings suggest one mechanism by which the organotropism of metastatic cancer cells can arise. (Cancer Res 2006; 66(12): 5981-4)

Introduction

Tumor metastasis proceeds via a number of different mechanisms. The establishment of true metastases, however, depends upon the ability of the seeded cells to survive within the new microenvironment. With respect to this, tumor dissemination can be aided by a number of factors, including proteins that interact with, modify, or proteolyze the local extracellular matrix environment; oncogenes that promote cell proliferation in the absence of growth factors; and factors that disrupt programmed cell death. These events can accumulate in established, nonmetastatic tumors, permitting nonmetastatic tumors to acquire a metastatic phenotype. One class of abnormalities, “metastasis suppressor genes” (reviewed in ref. 1), includes the disruption of genes that suppress cell migration and invasion, or alternatively those that promote apoptosis. Disruption of these homeostatic genes may significantly affect tumor spread.

In neuroblastoma, early epigenetic or oncogenic events in neural crest-derived cells, such as amplification of the N-MYC oncogene, are thought to initiate metastasis (reviewed in ref. 2). Other independent factors are associated with poor prognosis, including genetic alterations (LOH1p36) or expression of the growth factor receptors, such as TrkB. TrkB is of particular interest as it confers resistance to anoikis, a form of apoptosis that occurs when cells lose attachment to the underlying extracellular matrix. TrkB seems capable of conferring the necessary “survival signals” in lieu of cellular adhesion, thus sustaining cells that have either become dislodged or which have degraded the extracellular matrix in their immediate microenvironment (resulting in increased metastasis; ref. 3).

Anoikis is initiated by apical caspases in the apoptotic cascade, typically caspase-8 or caspase-9 (4). In neuroblastoma, caspase-9 activity is normally intact (5), but caspase-8 expression is selectively lost in aggressive disease (6). The reason for caspase-8 loss is not clear, but caspase-8 initiates the apoptotic cascade triggered following ligation of “death receptors” and therefore plays a key role in promoting the elimination of cells recognized as aberrant. Loss of caspase-8 can increase neuroblastoma survival following exposure to death receptor ligands, such as Fas, tumour necrosis factor-α (TNF-α; ref. 6), and TNF-related apoptosis-inducing ligand (7). “Resistance to death receptor–mediated apoptosis” therefore provides a rationale for caspase-8 loss, as tumor progression and metastasis require evasion of host defense responses.

On the other hand, the loss of caspase-8 is not the only mechanism that prevents death receptor–mediated killing. Critical downstream caspases can be inhibited via inhibitor of apoptosis proteins that are commonly overexpressed in tumors (8), whereas cytosolic proteins, such as c-FLIP or PEA-15, can compete with caspase-8 for binding to the death-inducing signaling complex, preventing apoptosis (9). Finally, the activation of oncoenic signaling cascades, such as those regulating the serine/threonine kinase Akt, also block caspase-8 activation and cell death (10).

Given the abundance of alternative mechanisms for controlling caspase-8-mediated death, coupled with recent observations that caspase-8 plays a role in cell migration and nuclear factor-κB activation (11), it may be advantageous for tumor cells to maintain caspase-8 expression. In fact, caspase-8 is required for survival during development (12), possibly explaining why alterations in caspase-8 expression have been reported relatively infrequently. Although loss of caspase-8 expression is reported in medulloblastomas (13) and colon carcinomas (14), it seems to occur with frequency only in small cell lung carcinoma (15), glioma (16), and malignant neuroblastoma (6). Given the range other epigenetic changes documented in neuroblastoma (2), we wondered what function the loss of caspase-8 served, and why it was so common in metastatic disease.

Caspase-8 Is a Metastasis Suppressor

We examined the effect of caspase-8 expression on neuroblastoma tumor growth and dissemination in a chick embryo xenograft model (17). The chorioallantoic membrane was permissive for the growth of all human neuroblastoma tumor cell lines tested, which contrasted markedly with our attempts to grow these tumors in immuno-suppressed adult mice. The chorioallantoic membrane model allowed rapid and simultaneous assessment of both initial tumor growth and metastasis to distant sites, such as bone marrow and lung. Caspase-8 expression was not a predictor of the capacity of neuroblastoma tumor lines to initiate tumors in these embryos (18), suggesting that caspase-8 was not generally a
tumor suppressor. However, expression of caspase-8 was associated with increased apoptosis among tissue-invasive tumor cells observed proximal to the tumor margin (although not within the tumor parenchyma), indicating that caspase-8 was regulating tumor survival during tissue invasion.

Collagen is the principle extracellular matrix constituent of the chorioallantoic membrane, and the pattern of survival among cells lacking caspase-8 and death among cells expressing caspase-8 were repeated in vitro within three-dimensional collagen gels. Thus, caspase-8-deficient cells were able to produce colonies in collagen gels, whereas neuroblastoma expressing caspase-8 were not. We noted that high-density seeding of neuroblastoma cells within the matrix offsets apoptosis (as individual cells quickly formed aggregates). In this milieu, caspase-8 expression did not affect survival, explaining why no difference was observed in initial tumor formation and growth in vivo.

The increased survival among the caspase-8-deficient cells reflected increased metastatic capacity. PCR detection of neuroblastoma cells in the lungs and bone marrow of the developing chick (as well as in the liver and distant sites in the chorioallantoic membrane) was possible within 1 week after seeding the tumor cells in the chorioallantoic membrane. Microscopic analysis of distal sections of chorioallantoic membrane confirmed the presence of perivascular colonies of tumor cells. The metastatic advantage of caspase-8-deficient cells was eliminated by reconstitution with caspase-8, indicating that caspase-8 expression is sufficient to block metastasis. Small interfering RNA–mediated knockdown of caspase-8 expression also increased the incidence of metastasis in caspase-8-positive neuroblastoma lines. This result was somewhat unexpected, because the caspase-8-expressing tumor lines used had already shown de facto metastasis in human patients, and it was assumed that these cells might, therefore, have altered caspase-8 activity by other mechanisms. The increase in metastasis of these lines strongly supported the notion that caspase-8 was a metastasis suppressor. We concluded that whereas the loss of caspase-8 is not required, it clearly potentiates metastasis.

Caspase-8 was absent in murine neuroblastoma that exhibit spontaneous metastasis in mice. Human neuroblastoma did not undergo spontaneous metastasis in the mouse, although experimental metastases could be generated several months after tail vein injection of a heavy (10^7 cells) tumor burden. All of the tumors analyzed displayed down-regulation of caspase-8 at both the RNA and protein levels, and caspase-8 remained absent in cell lines we established from these metastases. As expected, the metastatic neuroblastoma lines exhibited increased survival in vitro and increased metastasis in vivo. Caspase-8 expression could be restored in these cells by 5-azacytidine treatment, suggesting that at least part of the down-regulation of caspase-8 resulted from hypermethylation of the caspase-8 gene (6). This could have important implications for future treatment of disease in children, particularly if one considers combinatorial therapies that also promote caspase-8 activation.

Mechanisms of Caspase-8 Activation

The loss of caspase-8 was not simply to avoid death receptor–mediated killing. We found that reexpression of caspase-8 was typically insufficient to render cells susceptible to death receptor–mediated killing. Treatment with INF-γ and TNF-α (sufficient to initiate apoptosis in many cell types) or Fas did not typically kill neuroblastoma in the absence of cycloheximide (a protein translation inhibitor). These findings implicated additional factors in the inhibition of death receptor–mediated killing in neuroblastoma and suggested the loss of caspase-8 was redundant. These results reflect observations in other tumor types, where loss of caspase-8 is not required to prevent death receptor–mediated killing. However, we suspected that alternative caspase-8–mediated death pathways might be active for two reasons. First, caspase-8-dependent apoptosis occurred in vivo in the absence of inhibitors of protein translation, and second, it occurred in environments where compatibility between host “death ligands” (mouse and chick) and tumor cell death receptors (human) was difficult to show. Finally, the expression of dominant-negative forms of the death adaptor protein FADD, which block death receptor–mediated killing, did not promote metastasis or rescue caspase-8-expressing neuroblastoma from apoptosis.

We felt that the apoptosis we observed in vivo might was unlikely to result from anoikis for a number of reasons. First, the cells we observed dying were actively invading the collagen stroma, suggesting that they attached to and interacted with the extracellular matrix. Certainly, in vitro, all of the neuroblastoma cells showed moderate to strong adhesion to collagen coated surfaces. Second, we found that selection for neuroblastoma cells with decreased integrin expression actually promoted survival, which was inconsistent with the notion that “integrin-mediated survival signaling” was critical to avoid cell death in our system. Third, anoikis can be affected by either caspase-8 or caspase-9, and all of the neuroblastoma studied had a fully functional caspase-9 death pathway (5, 18). Thus, we did not believe we could explain caspase-8-dependent apoptosis via an anoikis mechanism.

However, we previously described a process, termed integrin-mediated death (IMD), by which unligated integrins initiate apoptosis selectively via caspase-8 (19). IMD occurs in response to the accumulation and apparent clustering of unligated or antagonized integrins on the cell surface. The clustered integrin complex then recruits caspase-8, which can become activated via “induced proximity,” leading to cell death (20). The probability of observing IMD in a given cell is a function of the local microenvironment around a tissue-invasive cell and the particular integrin “repertoire” expressed by that cell.

Significance of IMD in Disease

We previously suggested that integrins function as “dependence receptors,” requiring productive ligation to prevent an apoptotic response (21). Such a model would also explain why only those cells that exit the primary tumor undergo IMD (Fig. 1). Although ligation of a subset of integrins in the collagogenous environment may be sufficient to permit cell migration and invasion, it may nonetheless be insufficient to prevent IMD, particularly if ligands for other integrins (such as fibronectin and laminin) are limiting. In contrast, the initially seeded tumor offers high cell density, abundant cell surface integrin ligands, and extensive remodeling of the local extracellular matrix. This provides numerous opportunities for integrin ligation, and IMD is not observed. This may also explain why integrin expression and matched extracellular matrix ligand expression are common in aggressive tumors, as invasive cells deposit ligand for their own integrins (22).

IMD may provide a mechanism to explain why tumor cells seed specific environments better than others. For example, we observe

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Unpublished results.
integrin α4 expression commonly on neuroblastoma cells. Initially considered a hematopoietic integrin, this integrin is present on a number of metastatic cell types (23). Environments that provide ligands for this integrin, such as bone marrow, may be more likely to support cell survival, colonization, and the establishment of frank metastases (assuming the tumor cells do not express an abundance of other integrins that do not recognize this environment).

The elimination of caspase-8, or the depletion of integrins, may therefore benefit tumor cell survival during the invasion of a new tissue, particularly one for which their initial integrin receptor repertoire is poorly suited. The prediction arising from such a relationship would be that any metastases found in "unusual" sites would be expected to display a different integrin repertoire relative to those found in organotropic sites. This is testable, and there are already indications that cellular expression of different integrins will change preferred sites of metastases.

The loss of caspase-8 expression prevents IMD, providing one mechanism to escape apoptosis during tissue invasion. This provides an explanation for the disproportionate absence of caspase-8 in human patients with disseminated neuroblastoma relative to early stages of the disease (6). Human neuroblastoma cells also often exhibit decreased levels of integrin expression (24, 25), suggesting an alternative mechanism for decreasing IMD. This may be particularly important among neuroblastomas (and other tumors) that decrease but do not completely lose caspase-8 expression. For example, hematologic malignancies typically retain caspase-8 expression but generally express low levels of integrins. Consistent with this concept, overexpression of integrins can actually impair tumorigenicity (26). Many tumors simply produce appropriate extracellular matrix ligands to ligate their integrins (22), thereby circumventing IMD while providing additional opportunities for integrin-mediated survival signaling. The colonization of a site that endogenously satisfies the minimal requirement for integrin ligation may, therefore, explain the intrinsic organotropism of some tumors.

Finally, it is worth considering that many extracellular matrix products can be present as soluble agents that may serve as "antagonists" of integrin-mediated adhesion to the extracellular matrix proper. These extracellular matrix proteins (or more commonly, protein fragments) are typically present during angiogenesis and tissue remodeling and are often produced by tumors themselves. The presence of these soluble elements may, therefore, influence cellular decisions to live or die, as they affect the cell's integrin-based perception of its microenvironment. This can tip the cellular "rheostat" towards an apoptotic response and therefore holds promise in a number of clinical applications.

Figure 1. Putative roles for integrins and caspase-8 in regulating tumor cell survival and dissemination. Black lines, migration from the primary tumor by typical tumor cells (top right, yellow cell) or by cells harboring alterations in integrin or caspase-8 expression (bottom, brown cells). Restoration of caspase-8 expression (red arrow) promotes apoptosis and compromises metastasis.
Nevertheless, low levels of integrin antagonists can actually promote cellular migration, and further work is clearly warranted to elucidate the molecular mechanisms by which integrins and caspase-8 interact to mediate apoptosis or migration (13).

Nevertheless, if caspase-8 expression could be elevated by releasing the transcriptional block that occurs in neuroblastoma (and a number of other diseases), then these diseases might be more readily managed in the clinic (7). Our observations suggest directly that therapies that increase caspase-8 expression would be expected readily to elucidate the molecular mechanisms by which integrins and promote cellular migration, and further work is clearly warranted.

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